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# Determination of mercaptobenzimidazol and other thyreostat residues in thyroid tissue and meat using high-performance liquid chromatography–mass spectrometry

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**Abstract**

This paper describes a method for extraction of tapazol, thiouracil, methylthiouracil, propylthiouracil and mercaptobenzimidazol (MBI) from thyroid tissue. The solid-phase extraction procedure is optimized to obtain the maximum results for the main thyreostats including MBI. Different combinations of sample application, column conditioning and wash steps were tested. The analytes were extracted from the matrix with methanol. After solid-phase extraction they were derivatised with 7-chloro-4-nitrobenzo-2-furazan. Determination is carried out using liquid chromatography–electrospray mass spectrometry. The identification of the analytes was performed according to the final revision of the EU criteria (93/256/EC decision). The detection capability was 20 μg $kg^{-1}$ for all mentioned thyreostats. 

*Keywords:* Food analysis; Thyroid tissue; Mercaptobenzimidazol; Tapazol; Thiouracil; Methylthiouracil; Propylthiouracil

## 1. Introduction

Thyreostatic drugs are banned in the EU (directive 86/469/EC). They have been applied illegally to animals to obtain a higher live weight gain. This gain was mainly due to higher water retention in edible tissue and filling of the gastro–intestinal tract. The first effect gives a fraudulous higher weight (''water instead of meat'') which in turn leads to a reduction of the meat quality [1,2]. Consumption of meat contaminated with thyreostats has caused an increased incidence in Spain of aplasia cutis, a characteristic scalp defect [3].

Previously published methods for the determination of thyreostats do not include mercaptobenzimidazol. Through the European Union Reference Laboratory (CRL, RIVM, Bilthoven, the Netherlands) we were informed that a ''new'' thyreostatic compound was illegally used and not monitored. Multiresidue methods involve the use of TLC [4,5] and HPLC with UV detection [6]. Both methods lack specificity and selectivity but are useful for screening purposes. The identification criteria do not match the ones stated in the final version of the revision of the EC directive (93/256/EC) [7]. GC–MS methods have been described as suitable for confirmation [8–10]. Blanchflower et al. (1997) reported an LC–

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MS method using an atmospheric pressure chemical ionization (APCI) interface and selected ion monitoring (SIM) [11]. This paper mentions the detection of MBI with the LC–MS method but no mass spectrum or extraction data are provided.

Thin layer chromatography suffices for the determination of mercaptobenzimidazol (MBI, 2-benzimidazolethiol) in highly concentrated ''suspicious'' solutions seized at the farm. Confirmation of these concentrated solutions is performed with infusion-electrospray-MS [12]. A protonated molecular ion is formed at an intensity large enough to detect it above background ions. Further fragmentation provides enough information for a positive identification. Infusion MS can not be manipulated for a large number of samples. In between samples the tubing and spray shield have to be flushed extensively to prevent a memory-effect of the infused analyte. An operator has to be present during the whole of the experiment. Since an overnight procedure in a routine way is advisable, infusion was not an option.

Using the routine method based on the complex formation of the thyreostats with a mercurated resin MBI is lost [13]. The loss of MBI during this procedure is probably due to the interaction of the benzenering (Fig. 1) with the aromatic units of the resin. It was our goal to create or optimize an existing extraction method for thyroid tissue that included MBI.

The method reported in this paper detects the analytes in MS–MS-full scan. The precursor ion ($MH^+$) is isolated in the ion trap and fragmented to produce specific product ions. The probability of reporting false positive results decreases when working in full scan mode compared with SIM.

## 2. Experimental

### 2.1. Chemicals and clean-up column

The chemicals for extraction were of analytical grade. The solvents for preparation of the mobile phase were of HPLC-grade. Both were obtained from Merck (Darmstadt, Germany). 7-Chloro-4-nitrobenzo-2-furazan (NBD-Cl) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Buffer, pH 8, was made up of 94.5 ml of 0.2 *M* $Na_2HPO_4$ mixed with 5.5 ml of 0.2 *M* $KH_2PO_4$. The pH was controlled and adjusted to 8. The NBD-Cl solution (12.5 μ*M*) was prepared from 5 mg of NBD-Cl was dissolved in 2 ml of methanol. The solution was prepared fresh daily and kept in a cool dark place.

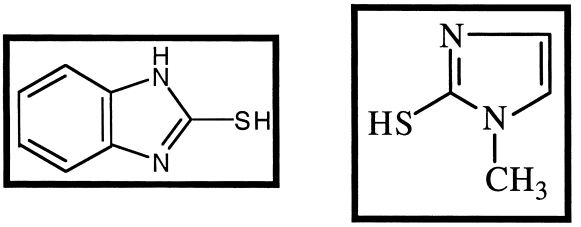


Fig. 1. Structural formula of MBI in comparison with tapazol.

For the clean-up step silica gel–SPE disposable columns (10 ml/500 mg) were used from IST (Mid Glamorgan, UK).

### 2.2. Standard solutions

Standards solutions were obtained from Sigma–Aldrich. Stock solutions of the thyreostatic drugs: 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 1-methyl-2-mercapto-imidazole (tapazol, TAP) and 2-mercaptobenzimidazol (MBI) were prepared in methanol at a concentration of 20 mg/100 ml (200 ng $\mu l^{-1}$). A working solution was obtained by 200×dilution in methanol (1 ng $\mu l^{-1}$).

The internal standard solution was composed of 20 mg of 4(5,6)-dimethyl-2-thiouracil (DMTU) dissolved in 200 ml of methanol (200 ng $\mu l^{-1}$). A working solution was obtained by 200×dilution in methanol (1 ng $\mu l^{-1}$).

### 2.3. Instrumentation

The HPLC apparatus comprised of a TSP P4000 pump and a model AS3000 autosampler (TSP, San Jose, USA). Separation was carried out on a Symmetry $C_{18}$ column (5μm, 150×2.1 mm, Waters, Milford, USA). Analysis was carried out using an LCQ ion trap mass analyzer (ThermoQuest, San Jose, USA), with an electrospray interface and Navigator 1.2. software. The analytes were detected in MS–MS-full scan positive ion mode.

To separate the different compounds, a linear gradient was used using a mixture of 45% methanol–55% of 0.73% acetic acid. The gradient started for 12 min with 100% of the previous mixture. At 22 min, the methanol composition increased to 100%.

### 2.4. Extraction and clean-up

Two grams of thyroid tissue were sliced into very thin pieces, and 10 ml of methanol were added to the samples. The samples were placed in an ultrasonic bath for 30 min and left to stand overnight at room temperature to allow the analytes to extract from the matrix. The samples were centrifuged for 20 min at 9000 rpm. The supernatant was filtered over a Whatman filter (no. 2,125 mm) (Springfield Mill, UK). To the filtrate 60 μl of a 1 ng $\mu l^{-1}$ internal standard solution (DMTU) was added. The samples were evaporated to almost dry in a speedvac (S210A, Savant Instruments, NY, USA) under vacuum. The remainder was evaporated to dryness under a gentle stream of nitrogen.

The residue was dissolved in 500 μl of chloroform, vortexed and left in an ultrasonic bath for a few seconds. Prior to application on the column 2.7 ml of *n*-hexane was added and the mixture was vortexed. For the clean-up procedure of the sample extracts, the silica cartridge was conditioned with 4 ml of *n*-hexane. The extract was applied onto the column. The column was washed with 4 ml of *n*-hexane. The thyreostats were eluted with a mixture of 2.5 ml of 15% methanol–85% chloroform, which was evaporated to dryness under a slow stream of nitrogen.

### 2.5. Derivatisation

Five millilitres of phosphate buffer, pH 8, were added and the eluate was neutralized and adjusted to pH 8. A methanolic NBD-Cl solution (0.1 ml) was added and the reaction was allowed to proceed in the dark at 40°C over 1 h. Thereafter, the reaction mixture was adjusted to pH 3–4 by adding 0.2 ml of 6 *M* HCl. The NBD-derivatives were then extracted successively with 3, 2 and 2 ml of diethyl ether. The combined ether extracts were dried over sodium

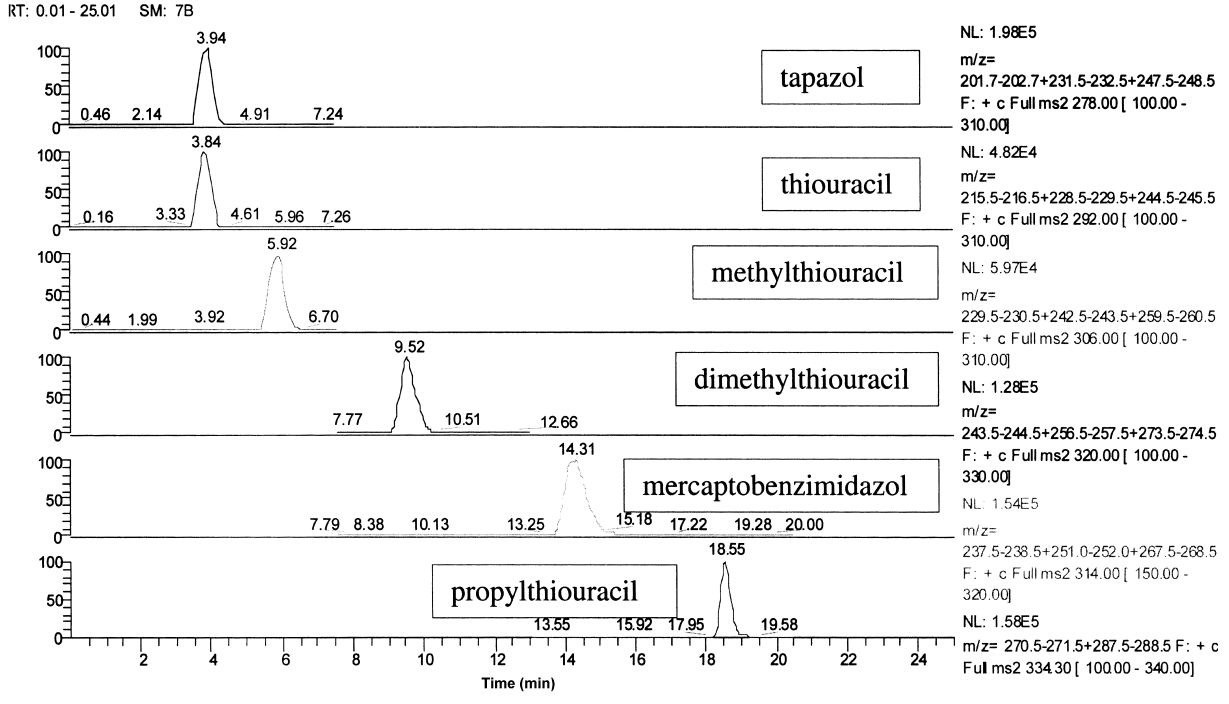


Fig. 2. Chromatogram of a standard mixture at 2 ng on the column.

sulphate and evaporated to dryness under a stream of nitrogen.

The residue was dissolved in 30 μl of ethanol, vortexed and 70 μl of mobile phase was added. Of this extract, 30 μl were injected onto the column. The extracts are analysed the same day of the derivatisation. With each batch of samples a standard was acquired. For the preparation of the standard stock derivatives, 20 μg of all analytes were derivatised. The derivatives are dissolved in 1 ml of ethanol after evaporation.

The final concentration of this derivative stock solution was 20 ng $\mu l^{-1}$. Working solutions are prepared in mobile phases at 2 and 0.2 ng $\mu l^{-1}$. Aliquots of 10 μl of a 0.2 ng $\mu l^{-1}$ mixture were injected onto the column to check the retention times of the analytes and if necessary to correct the division of the segments. The stock derivatives (20 ng $\mu l^{-1}$) were stable for 1 month. The working solutions in the mobile phase, however, were prepared with each new batch of samples.

### *2.6. Detection*

The elution order of the thyreostats was as follows: thiouracil, tapazol, methylthiouracil, dimethylthiouracil, mercaptobenzimidazol and propylthiouracil. (Fig. 2.). The time axis was divided into four time segments. In the first segment three analytes were scanned consecutively. The pseudomolecular ions of TAP, TU and MTU were fragmented and a full scan product ion spectrum was acquired. In the second segment product ions of DMTU and MBI were generated. In the third segment MBI and PTU were fragmented.

Because of possible retention time shifts due to temperature variations or column deterioration, we did not provide a separate segment for MBI. By acquiring it during the two segments no datapoints for MBI were lost. The fragmentation characteristics are given in Table 1.

The analytes of which the most abundant daughter ion was formed through loss of $NO_2$ (−46) do not contain a hydroxyl group in their original structure (TAP). Thyreostats related to thiouracil form stable intense daughter ions through the loss of $NO_2$ and OH (−63).

## 3. Results and discussion

The extraction procedure published by Blanchflower et al. (1997) was used as a starting point for the optimization for the extraction of the six thyreostats from thyroid tissue. The column was conditioned with chloroform. The sample was applied to the column in chloroform and the cartridge was also washed with chloroform. The analytes were eluted with methanol–chloroform (15:85, v/v).

When applying this strategy we did not obtain good results. In a first stage the procedure was tested without a matrix able to collect each step and analyze it without further clean-up. Different combinations of column preconditioning, sample application and wash steps were tried. The combinations are summarized in Table 2. The results of the different combinations are given in Table 3. Each step of the SPE-procedure was collected and analyzed. Each combination, which gave a positive response for one analyte in the wash step or the percolate, was discarded. For three combinations (8, 9, 10) only the eluent was positive, the wash step or percolate gave no response. The absolute peak areas were compared and the combination with the highest response was

Table 1
Fragmentation characteristics of seven thyreostats

| Analyte | Precursor ion | Collision energy (%) | Product ions |
|---|---|---|---|
| Tapazol | 278 | 23 | 202,232,248 |
| Thiouracil | 292 | 22 | 216,229,245 |
| Methylthiouracil | 306 | 22 | 230,243,260 |
| Dimethylthiouracil | 320 | 25 | 244,257,274 |
| Mercaptobenzimidazol | 314 | 25 | 238,251,268, 284 |
| Propylthiouracil | 334 | 27 | 271,288 |

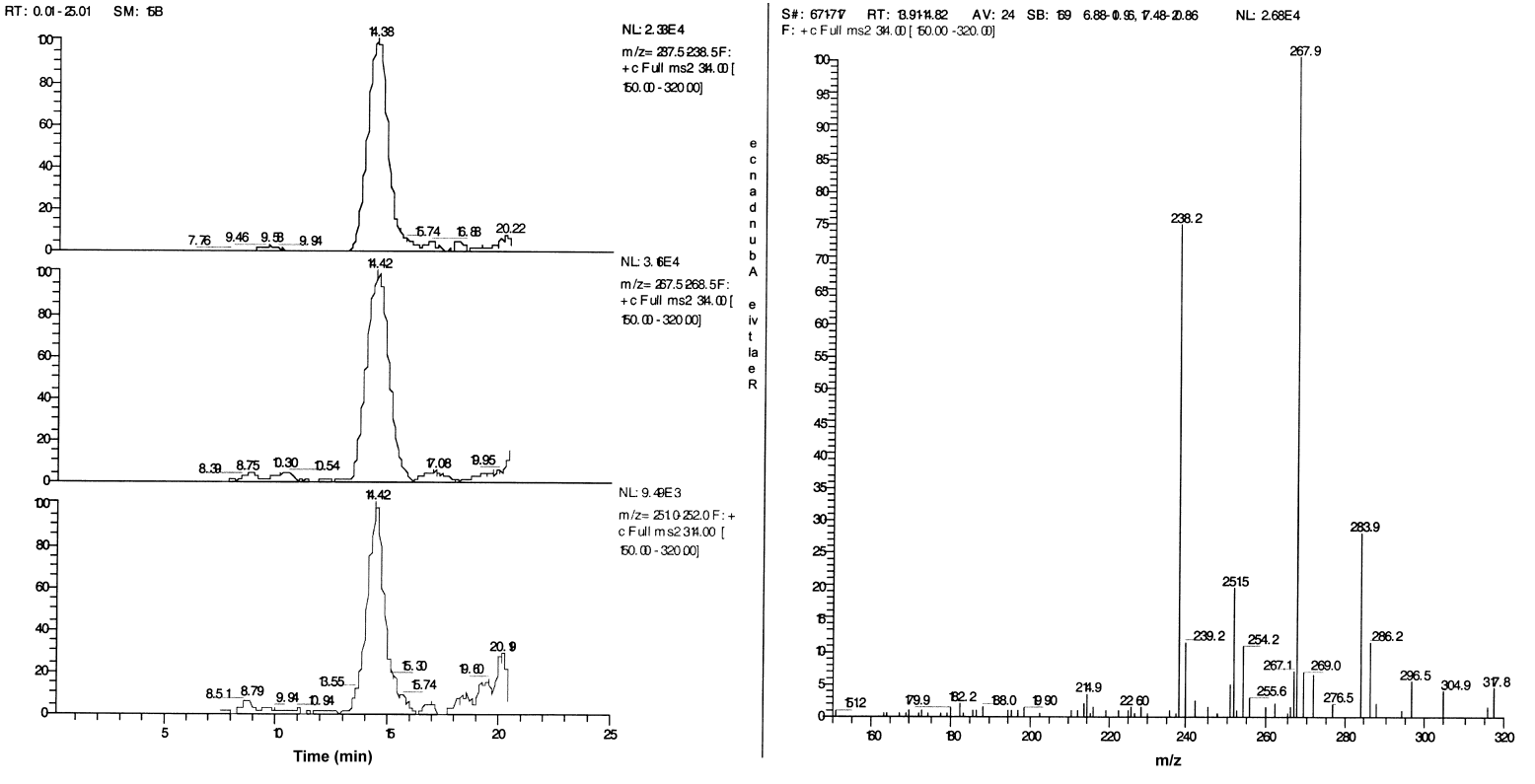


Fig. 3. Ion chromatogram and spectrum of MBI in matrix at 20 μg $kg^{-1}$.

Table 2
Different combinations for preconditioning of the SPE cartridge, sample application and wash steps

| Preconditioning SPE | $CHCl_3$ | | | | $CHCl_3$ | | | *n*-Hexane | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Sample application[a] | 100/0 | 90/10 | 50/50 | 10/90 | $CHCl_3$ | | | 10/90 | 50/50 | 90/10 |
| Wash solvent[a] | CHCl3 | | | | 90/10 | 50/50 | 10/90 | *n*-hexane | | |
| Elution | MeOH–$CHCl_3$ (15/85) | | | | | | | | | |
| Experiment number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

[a] $CHCl_3$/*n*-hexane.

Table 3
Positive response for the different combinations

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Tap | e, w | e, w | e | e | e, w, p[a] | e, w, p | e, w, p | e | e | e |
| MTU | e, w | e, w | e | e | / | / | / | e | e | e |
| TU | e | e | e | e | / | / | / | e | e | e |
| PTU | e, w | e, w | e, s | e, s | e, w, p | e, w, p | e, w, p | e | e | e |
| SMTU | e, w | e, w | e | e | e, w, p | e, w, p | e, p | e | e | e |
| MBI | e, w | e, w | e, s | e | e, w, p | e, w, p | e, w, p | e | e | e |

[a] e, w, p=Positive response eluent, wash step, percolate.

tested with the matrix. Combination 8 proved to give the best results. Initially it was our goal to first screen the samples with TLC and only analyze the suspect samples with LC–MS–MS. The limit of detection for the TLC plates was too high. Too many false negative results would escape the routine surveillance program.

Working in MS–MS-full scan allows us to be very specific. Fragmenting the thyreostats without derivatisation produces ions with a low $m/z$ value as illustrated by Blanchflower et al. (1997). A disadvantage of the lower mass range is the increased chemical noise. Working in CID–MS and acquisition in SIM can slightly overcome this problem but is not an option in ion trap technology. Ion traps give the best results working in full scan. Derivatising the analytes brings the molecular weight in a higher mass range with less background interference. The fragment ions fulfil the criteria of the final version of the revision of the EU criteria (93/256 EC) as stated in Table 4. As an example we compared in Table 5 the intensity ratio of one diagnostic fragment ion to the most intense fragment ion, between the standard injection of a batch of samples and the spike sample. The maximum tolerance levels were calculated dependent on the intensity of the ion. Since all intensities of the ion with $m/z$ 238 were situated above 50% relative intensity to the base peak, a tolerance window of 20% was allowed and fulfilled. Whenever a positive sample occurs the same approach will be applied.

Next to the criterion for the tolerance levels we

Table 4
Maximum permitted tolerance for relative ion intensities using a range of mass spectrometric techniques

| Relative intensity (% of base peak) (relative) | EI–GC–MS (relative) (±%) | CI–GC–MS, GC–MS–$MS^n$ LC–MS, LC–MS–$MS^n$ (±%) |
|---|---|---|
| >50 | 10 | 20 |
| >20–50 | 15 | 25 |
| >10–20 | 20 | 30 |
| ≤10 | 50 | 50 |

Table 5
Calculation of the maximum permitted tolerance levels of MBI product ion 238 for a spike sample at 20 μg $kg^{-1}$ and a standard injection of 2 ng on the column

| Intensity (%) | Product ion 238 | 268 | Lower limit | Upper limit |
|---|---|---|---|---|
| Spike 1 | 72 | 100 | 58 | 87 |
| Standard 1 | 77 | 100 | 62 | 93 |
| Spike 2 | 69 | 100 | 55 | 83 |
| Standard 2 | 67 | 100 | 54 | 80 |
| Spike 3 | 68 | 100 | 54 | 81 |
| Standard 3 | 59 | 100 | 47 | 71 |

Table 6
Relative retention time windows for the different thyreostats (average±3×standard deviation)

| Analyte | Lower limit | Average | Upper limit |
|---|---|---|---|
| Tap | 0.29 | 0.38 | 0.48 |
| TU | 0.29 | 0.39 | 0.49 |
| MTU | 0.55 | 0.60 | 0.66 |
| PTU | 1.55 | 1.94 | 2.33 |
| MBI | 1.46 | 1.49 | 1.52 |

also add criteria for the relative retention time. For each analyte a relative retention time window is applied that is calculated based on the retention of spike samples and standards. The retention time windows are mentioned in Table 6.

Different labs use different approaches for the determination of detection limits. In our lab we use the detection capability. This is defined as the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of $1-\beta$ (error probability=5%). The detection capability is based on the analysis of 20 blank samples fortified at the level of 20 μg $kg^{-1}$. All analytes were detected in 19 out of 20 fortified samples. This means they were detected with a statistical certainty of 95%. In that case the detection capability is far below the demands of the European Union (100 μg $kg^{-1}$). A detection limit can be given corresponding with a signal-to-noise ratio of 3 and is for the different thyreostats 1, 1.5, 1.3, 1.1, 0.9 and 1.9 μg $kg^{-1}$ of tapazol, thiouracil, methylthiouracil, dimethylthiouracil, propylthiouracil and mercaptobenzimidazol, respectively.

## 4. Conclusion

The optimized extraction procedure allows the detection of all thyreostats including MBI up to a level of 20 μg $kg^{-1}$ (Fig. 3). The detection of all thyreostats in spiked thyroid tissue with LC–ESI–MS–MS is unambiguous. Good specificity is obtained working with full scan mass spectra of the product ions. Specificity criteria were demonstrated and found adequate for a positive identification.

Other matrices (urine, feces, feed) are tested with this method but further development is needed.

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